Protein A Chromatography Resin Lifetime - Impact of Feed Composition

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Abstract

Adsorbent lifetime during protein A chromatography is not readily predicted or understood, representing a key challenge to be addressed for biopharmaceutical manufacturers. This paper focuses on the impact of feed composition on the performance of a typical agarose based protein A resin across a lifetime of 50 cycles. Cycling studies were performed using three different feed materials with varying levels of feed components including proteases, histones, DNA, and non-histone proteins. Changes in the process and quality attributes were measured . The DBCs were not seen to vary between conditions although there was a reduction in particle porosity in all cases. Fluorescence spectroscopy and LC-MS/MS were used to identify the contribution and extent of fouling to the observed capacity loss. Residual protein A ligand density and deposition of foulants (HCP, residual mAb and DNA) varied between the three feed materials. Resins cycled in feed materials containing high concentrations of HCP and histones were seen to have greater extents of capacity loss. The mode of performance loss, capacity loss or impact on product quality was seen to vary depending upon the feed material. The results indicate that feed material composition may be correlated to the rate and mode of resin ageing as a basis for improved process understanding.

***Key words**: Protein A chromatography, resin lifetime, feed material components, fluorescence, LC-MS/MS*

1. 1 Introduction

Protein A chromatography is widely used for purification of monoclonal antibodies and Fc fusion proteins [1-3]. Owing to the high cost of materials, it is common to reuse protein A chromatography media [1,4]. The impact of reuse can have on product recovery, product quality, and the clearance capacity is well documented [3,5]. To avoid performance loss during protein A chromatography cycling, manufacturers often use sodium hydroxide for cleaning as it has the ability to hydrolyze proteinaceous residues and simultaneously sanitize the resin [6-8] yet the maximum number of times a protein A resin can be reused remains variable, ranging from 50-200 cycles [4]. Although several mechanisms have been proposed in the literature that can contribute to chromatographic performance

loss [9], identification of a predictive parameter for fouling remains elusive. While there have been cases where a process has been successfully operated for an extended number of cycles (>100) without major loss of performance, there have been others where substantial resin fouling occurs even after a relatively small number of cycles [4,10]. In such cases, the ability to monitor accumulation of foulants within the resin and on the resin surface is critical in order to assess effectiveness of cleaning protocols or to develop strategies to prevent fouling.

In addition to monoclonal antibodies (mAbs), the clarified cell culture broth obtained from CHO cells contains various biological impurities including host cell proteins (potentially important components in this class are histones and proteases) DNA, and lipids. Histones have recently received particular attention as a causative agent in protein A chromatography performance loss. It is proposed chromatin binds to protein A by strong nonspecific electrostatic and hydrophobic interactions through the histone component. Chromatin binding to protein A is likely to result in two negative outcomes; reduced accessibility to the Fc-specific binding sites and thereby reducing IgG capacity, and reduced product purification due to co-eluting or leaching subsets of process impurities during IgG elution [11-15].

This paper aims to examine the impact that feed composition has on protein A chromatography resin capacity loss. We expect the results presented here to be of interest to those working on purification of monoclonal therapeutic products.

2. Materials and methods

2.1 Materials and equipment

Three different CHO feeds containing three different human IgG monoclonal antibodyies (mAb) with isoelectric points between 7.3 to 8.9 and molecular weight ~150 kDa, were donated by a major Indian manufacturer of biosimilars. The feed material consisted of clarified harvest with product concentration of 1 to 4 mg/ml. Viability at harvest for H1 was 73.5%, 90% for H2 and NLT 40% for H3. All feed material was centrifuged at 6000 rpm for 15 minutes and applied to a 0.2 μm filter prior to loading onto the column.

All chromatographic experiments were carried out on an Äkta purifier (GE Healthcare, Uppsala Sweden) chromatographic system. This system has built-in UV, pH, and conductivity detectors to monitor the effluent from the chromatographic experiments. Empty Tricorn glass columns with 0.5 cm I.D. were procured from GE Healthcare Life Sciences (Uppsala, Sweden). MabSelect SuRe™ resin was also procured from GE Healthcare (Uppsala, Sweden). ForteBio Octect® RED 96 system from Pall Life Sciences (California, USA) was used for impurity detection and Agilent (California, USA) HPLC 1200 system was utilized for aggregate analysis.

Sodium chloride, sodium dihydrogen phosphate, and disodium hydrogen phosphate buffer salts were purchased from Merck, India. For preparative chromatography, analytical grade chemicals were used and for analytical chromatography, HPLC grade chemicals were used. Blue dextran was purchased from GE Healthcare Life Sciences (Uppsala, Sweden).

2.2 Methods

2.2.1 Scale down model for resin cycling

Cycling studies were performed using the Mabselect SuRe™ resin packed column (5 cm x 0.5 cm) and protein A chromatography was scaled down as described in our previously published protocol [16]. A single cycle comprised the following steps: milli-Q wash, equilibration, loading, washing, elution, and stripping. The column was equilibrated with a 25 mM Phosphate, 50 mM NaCl, pH 6.2. Post equilibration, a minimum of five CV equilibration buffer was passed through the column. This was followed by loading of the clarified cell culture broth (CCCB) at a loading capacity of 15 mg/ml of resin. The column was washed with a minimum of 5 CV equilibration buffer after loading. Elution was performed using 100 mM acetate, pH 3.5 buffer, and was followed by regeneration with 2 M NaCl and cleaning with 50 mM NaOH, 1 M NaCl (contact time less than 15 minutes). The mobile phase velocity was 200 cm/h.

Cleaning was performed after every third cycle. In cycling studies without feed material, all steps were as previously described with the exception of the loading step. Robustness of the scale down model was established by performing 40 cycles in duplicates as per the protocol defined above. Similarly,

resin cycling studies with and without product loading were performed using the protocol mentioned above.

2.2.2 Estimation of dynamic binding capacity

DBC at 10% of the breakthrough curve was determined for the fresh resin (before starting column cycling studies) and after every 10 cycles. Purified mAb was used to determine DBC [16]

2.2.3 Impurity analysis

Aggregate content in the eluate samples was analyzed using a Superdex 200GL size exclusion chromatography column from GE Healthcare (Uppsala, Sweden), with 25 mM phosphate buffer at pH 6.8 and 300 mM NaCl in isocratic mode at a flow rate of 0.5 ml/min for 45 minutes. Histone HCP concentration was estimated using EpiQuik Total Histone Quantitation kit (Epigentek Inc., NY, USA). Histones were extracted from cycled resin and elute by 16 h (overnight) incubation in 200 mM HCL, pH 1.0, before centrifugation at 13,000 rpm for 10 min at 4°C. Supernatants were applied to histone ELISA plates, also in 200 mM HCL pH 1.0, and ELISA was performed as per the manufacturer's recommendations. HCP content was estimated using Pall ForteBio LLC and Cygnus Technologies jointly developed 3G, Anti-CHO HCP Detection Kit for quantitation of residual HCPs. Sample preparation for DNA analysis was conducted by proteinase K digestion (2 mg/mL proteinase K in 5% SDS added 1:9 to samples) for 16 h at 50°C, followed by DNA extraction using either a DNA extractor kit (Wako, P/N 295-50201) or using phenol/chloroform extraction method and the DNA content measured using picogreen dye. Total protease activity of the samples was determined by the Fluoro™ Protease Assay using the Fluorescent Protease Assay kit from G-Biosciences.

2.2.4 LC-MS/MS analysis

The LC-MS/MS analysis were performed using C18 Acclaim PepMap100 (Thermo, UK) 75 μm internal diameter x 15 cm (C18, 3 μm, 100 A) reverse phase HPLC column (Nano LC Ultimate3000, Thermo UK) using our previously published protocol [10]. MALDI-TOF-TOF was conducted using

an UltrafleXtreme MALDI-TOF instrument (Bruker, Coventry, UK) in positive ion reflector mode and 50% laser power and MS-MS was conducted on the ten most intense peaks for each target spot.

2.2.5 Estimation of mass transport parameters

Inter-particle porosity ($\varepsilon_p = V_{Dex}/V_{col}$) was determined by performing 100 □00as □□□□□□□□□□□□□□□□□□□□□□□□□/ml^{-1} blue dextran solution (molecular weight of 2×10^6 Da). Further, a pulse injection of 100 µL of 1 M NaCl was used to determine the total porosity. The intraparticle porosity ($\varepsilon_i=(V_{NaCl}-V_{Dex})/(V_{col}-V_{Dex})$) was calculated from these two values[16].

2.2.6 Electron microscopy imaging

Resin sample preparation and imaging were performed for fresh and fouled resin sample at the end of 50th cycle using our previously published protocol [10]. Images were obtained using a Jeol JSM-7401F field emission scanning electron microscope (Jeol Ltd, Tokyo, Japan). Parameters for image acquisition were 2 kV accelerating voltage and 5.0 µA, with a working distance of 5 mm.

2.2.7 Fluorescence analysis

The resin samples were monitored for foulant (HCP and residual mAb) deposition and ligand degradation by recording excitation and emission spectra at 250 nm to 500 nm. Direct real-time measurement of foulant accumulation on the resin could facilitate implementation of appropriate cleaning conditions to clear the deposited foulants[18]. Recombinant protein A contains tyrosine and phenylalanine residues but lacks tryptophan. However, host cell proteins and mAb do contain tryptophan as well as tyrosine and phenylalanine (as detected using LC-MS/MS). Consequently, a fluorescence intensity at 340 nm (due to the presence of tryptophan) can be associated with the foulants present on the resin, while the fluorescence intensity at 303 nm can be attributed to the protein A ligand present on the resin. We have evaluated and validated that the emission lambda maxima for protein A ligand bound to porous agarose resin was 303 nm while the mAb bound to protein A agarose resin was ~340 nm[19].

The light emitted by the light source was transferred to the slit and polarizer through the excitation monochromator. The slit and polarizer transmits light of a predefined wavelength to the emission monochromator. The predefined wavelength emitted by the emission monochromator was passed through the resin sample thereby resulting in the recording of fluorescence intensity by the detector. The fluorescence intensity was recorded for fresh resin and fouled resin at 50th cycle[18,19]. Similarly, to estimate the extent of DNA deposits with resin reuse, fresh and fouled resins were incubated with picogreen dye solution (diluted in TE buffer) and the spectra recorded using excitation at 480 nm.

Protein A leachate analysis

Protein A leachate was measured in the eluate from the 1st and 50th chromatographic cycle using Dip and ReadTM Residual Protein A Detection Kit (Pall Life Sciences, Portsmouth, UK) with an Octet Red 384 instrument (ForteBio, Pall Life Sciences, CA).

Results and Discussion

3.1 Characterization of feed material components

All three feed materials were derived from CHO cell lines and the cell viability for H1, H2 and H3 at the time of the harvest was 73.5%, 90% and 40%, respectively. The mAb concentration in the clarified cell culture broth (CCCB) was measured using protein-G HPLC and the contribution of other moieties to feed material composition determined. Separate kits were used to measure histones and non-histone proteins, as it has been reported that histones remain undetected if typical HCP quantitation ELISA kits are used [13]. Table 1 summarizes composition of the three different feed materials used in this study. HCP concentration was similar for the three feed materials. Notable observations were that H1 had high mAb, low DNA, intermediate histone and low protease concentrations. Feed material H2 had low mAb, very low histone, high DNA and relatively high protease concentrations. Finally, H3 contained low mAb, high histone, moderate DNA, and moderate protease concentrations. Thus it can be concluded that the composition of the feed materials vary, permitting a comparison to be made between feed material composition and the observed process and quality attributes of the chromatography resin.

3.2 Changes in process and product quality attributes during cycling of protein A resin

Cycling studies were performed for 50 cycles with each feed material. $DBC_{10\%}$ was measured at every 10^{th} cycle and the change in $DBC_{10\%}$ with cycle number is shown in Figure 2. It is seen that in all cases, DBC continues to fall up to the 50 cycles and reaches about 65% of the original value, indicating that considerable loss of capacity of the protein A resin occurred during the study period. This could be a result of ligand leaching, ligand degradation, coating of impurities on resin surface thereby reducing accessibility to the ligand, and/ or blocking of pores of the resin particles, all resulting in reduced accessibility to the ligand [16].

Levels of protein A leachate and proteases were also measured in the mAb elute. It was observed that protein A leachate was low (~100 ng/ml) in all three feed materials and the protease concentration fell below the accurate detection limit. This indicates that proteases may not be responsible for leaching during elution, although the possibility of protease-mediated protein A degradation during the loading steps cannot be eliminated. It has been suggested that relatively strong interactions between protein A ligand and mAb may lead to coelution of protein A ligand with IgG during the elution step [7,17,18], which may contribute to diminished ligand concentration on the resin in a mAb-dependent manner.

Protein A elute samples were taken for the 1^{st} and 50^{th} cycles and analyzed with SE-HPLC to determine the monomer content of eluted mAb form each feed material. Different fractions for aggregate and monomer peak were collected and analyzed by ELISA for the presence of histone. The monomer content in H2 was >99% for elute samples measured every 10 cycles without any increase in aggregate content (Figure 3B). However, in H1 the aggregate content increased from 12% to 16% by the end of the 50^{th} cycle, with three aggregate variants observed (Figure 3A). The histone content in each aggregate fraction was also found to have increased after 50 cycles. The aggregate content also increased in H3, from 0.8% to 7.6%, with a single aggregate variant peak (Figure 3C), and histone concnetraion within monomer and aggregate fractions also increasing. This data would suggest that the presence of histones in the protein A eluate can be linked with hitone content of the feed material, leading to a negative effect on product quality.

3.3 Accumulation of impurities on cycled resin

Particle porosity was monitored every 10 cycles and it was seen that particle porosity decreased with resin reuse to variable degrees with the three feed materials (Figure 3). The particle porosity decreased from 0.92 to 0.61 (~34% decrease) in H1, 0.92 to 0.72 (~22% decrease) in H2, and 0.93 to 0.51 (~45% decrease) in the H3. The inter-particle porosity remained unchanged at 0.45 after 50 cycles for all feed materials. These observations support the hypothesis that intra-particle resin pores become blocked as a result of fouling with column reuse [16] The extent of pore size reduction appears to correlate with the histone concentration in the feed material; H2 exhibited both the lowest histone concentration and the minimum decrease in porosity, followed by H1, and finally H3 showing the largest decrease in particle porosity and the highest histone concentration in the (Figure 3 and Table 1).

Figure 4 presents the fluorescence data for resin samples before and after cycling. The fluorescence intensity at 303 nm (expected peak when protein A ligand is targeted) was greatest for fresh resin followed by cycled resin samples from H1, H3 and then H2 feed materials. The lower intensity observed for H2 suggests that there was maximum degradation or loss of the protein A ligand in this sample, followed by H3 and H1. This theory corresponds with the feed components data presented in Table 1, where the maximum protease concentration is observed for H2, followed by H3 and then H1.

Fluorescence intensity at 340 nm (indicative of total non-protein A concentration) was greatest for H3 followed by H1 and H2, which signifies greater impurity deposition with H3 after 50 cycles. The intensity curve at 340 nm for H2 is very similar to that of the fresh resin, indicating minimal foulant deposition with H2. To further explore these observations, resin samples were digested and supernatants analyzed with LC-MS/MS. Figure 6 depicts the relative abundance of protein A, mAb and HCP residual on resin cycled with each feed material. The overall trend in residual protein concentration supports the results obtained from fluorescence analysis, namely that more material had accumulated on resin cycled with feed H3, then H1 and the least material being found on H2. The concentration of residual protein A was found to vary little between the feed materials when analyzed with LC-MS/MS, contrary to results derived from fluorescence analysis; this could perhaps be accounted for by less than 100%

efficient removal of all proteinaceous material prior to LC-MS/MS. It is also to be noted that although protein A is likely to be by far the most abundant protein which will emit at 303 nm, other residual proteins (mAbs, HCPs) will also contribute to the emission spectra.

To qualitatively determine the presence of DNA on the resin sample, fresh and cycled resin samples were incubated with picogreen dye and measured for DNA presence by exciting the sample mixture at 480 nm and recording the emission intensity at 520 nm. DNA content was found to be higher in H3 followed by H1 and H2. It is seen that cycled resin from H1 and fresh resin have similar emission spectra showing minimal deposition of nuclear content on the resin. This is logical as H1 feed contained the least DNA (Table 1). It has been shown that chromatin binds to protein A by nonspecific electrostatic and hydrophobic interactions through its histone component [11-13]. The strong association of DNA with histones can explain the apparent paradox of DNA binding to protein A despite the fact that purified DNA is electrostatically repelled by protein A. To further explore the significance of histones to resin fouling, cycled resin samples were digested using published protocols and analyzed by histone ELISA [11]. The histone content in the resin digest for H1, H2 and H3 were measured to be 3.8 µg/ml of resin, 0.4 µg/ml of resin, and 9.4 µg/ml of resin respectively (Table 3). These findings correspond to the trend in total residual HCP detected by LC-MS/MS, but do not follow the observation in histone concentration in the protein A eluate (Table 2). This indicates that high histone concentration in the feed cannot account for both an increased presence in the eluate and residual on the resin surface, but rather other factor(s) contributes to determine the role histones may play in capacity loss.

3.4 Mode of capacity loss

SEM images of resin cycled with each feed material demonstrate the physical manifestation of capacity loss also varies by feed material composition, in addition to the product quality attributes. The images shown in Figure 6 are representative of the observations made for each resin sample, although variation was inevitable likely due to the relative location of each resin bead within the column. Nevertheless, it was apparent that H3 showed the greatest visual signs of impurity accumulation, H1 displaying less extensive signs of material accumulation, and H2 displaying very little evidence of residual material

accumulation on the resin surface. These observations follow results from impurity analysis by fluorescence and LC-MS/MS, as well as the trends observed in particle porosity studies and DBC. The full picture of resin capacity loss is however more complex than impurity accumulation alone, as evidenced by the lack of a simple correlation between HCP concentration in the feed (Table 1) and residual HCP on the resin (Figure 6); although H1 contained the highest mAb, HCP and overall protein concentration, it was H3 which displayed the most severe signs of fouling-mediated capacity loss. SEC analysis reveals that H1 formed the most aggregates, and analysis of histone content within protein A eluate samples showed that not only were histones co-eluting with mAb but were also being enriched relative to other HCP species (Figure 2, Table 2). It could therefore be inferred that histones in the feed material pose a threat to protein A chromatography processes, a phenomenon reported previously by others (Gagnon). The extent and nature of the risk posed may depend on the interaction between histones and mAb; in this study, high mAb titer combined with high histone concentration in feed H1 was seen to manifest as increased product aggregation and histone:mAb coelution. When cell viability in the feed was low, as in H3, histone concentration was also elevated, but mAb titer was lower, thus histones primarily contributed with other HCP to blocking of resin pores, resulting in fouling-mediated capacity loss. Under our experimental conditions, when feed material contained lower mAb titer and lower HCP (including histone) concentration, the extent of capacity loss was much reduced, as exemplified by H2.

4. Conclusion

Maximizing the useful lifespan of preparative protein A columns is an important concern for the biopharmaceutical industry. A number of factors have been linked to performance degradation with resin reuse including resin fouling resulting in blockage of access to ligands, resin pore structure degradation, and ligand degeneration, ultimately resulting in a loss of binding activity. This study provides evidence for multimodal routes to capacity loss – fouling, ligand loss, product degradation or a combination thereof. The results obtained in this work suggest that feed material components play a significant part in determining this mode of capacity loss and so ultimately impacting column performance and product quality.

The presence of histones in the feed had an adverse effect on resin lifetime. We observed the presence of histones in the resin digest and the protein A eluate in H3 and H1, and suggest that histone proteins contributed to the observed decrease in capacity. The use of fluorescence analysis suggests that the decrease in resin capacity with H2 was primarily due to ligand depletion, possibly attributed to degradation during column loading by proteases present in the feed material.

The results presented in this paper are anticipated to assist in the development of robust and economical manufacturing processes for a wide variety of therapeutic antibody products.

2. Acknowledgements

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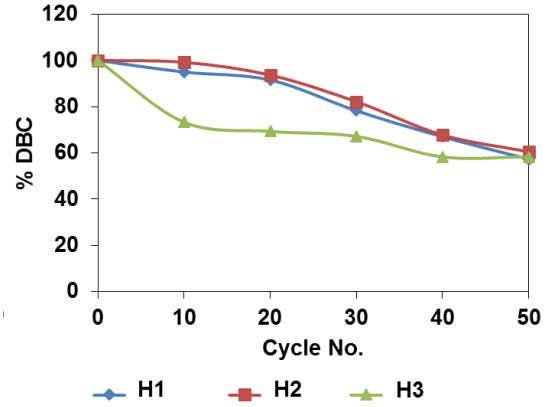
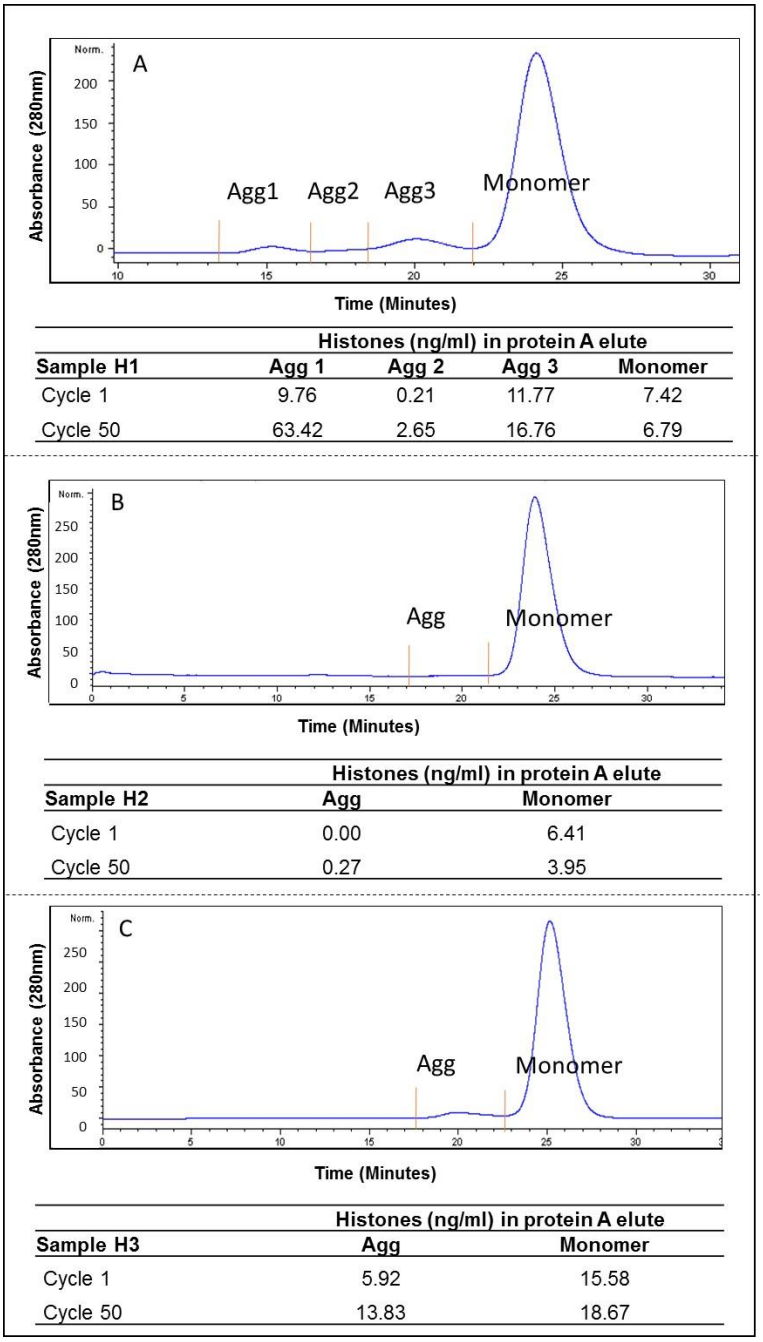


Figure 1: DBC at 10% breakthrough for H1, H2 and H3 feed materials for 50 cycles. DBC was determined every 10th cycle for each feed material.



	Histones (ng/ml) in protein A elute			
Sample H1	**Agg 1**	**Agg 2**	**Agg 3**	**Monomer**
Cycle 1	9.76	0.21	11.77	7.42
Cycle 50	63.42	2.65	16.76	6.79

	Histones (ng/ml) in protein A elute	
Sample H2	**Agg**	**Monomer**
Cycle 1	0.00	6.41
Cycle 50	0.27	3.95

	Histones (ng/ml) in protein A elute	
Sample H3	**Agg**	**Monomer**
Cycle 1	5.92	15.58
Cycle 50	13.83	18.67

Figure 2: SEC profile illustrating aggregates peak fractions for the protein A eluate. Aggregate samples were also assayed for histone content after the 1st and 50th cycle for H1, H2 and H3 feed materials.

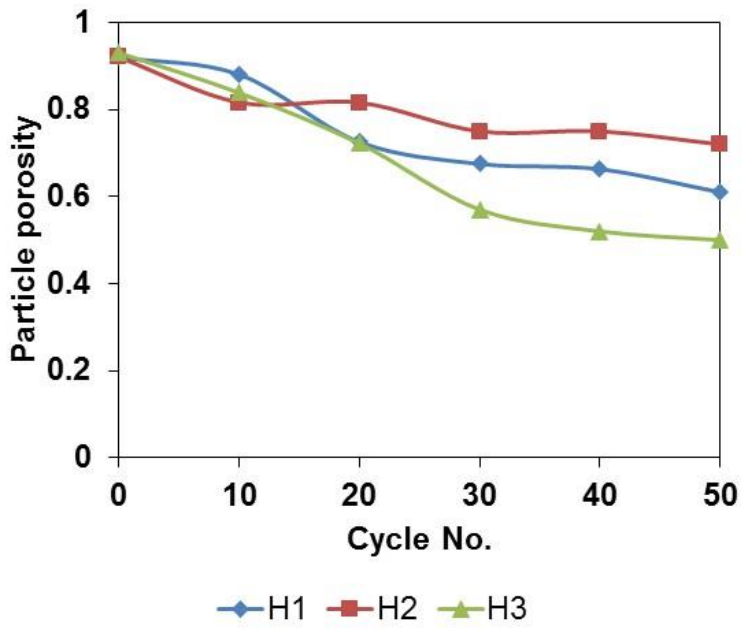


Figure 3: Comparison of particle porosity for feed materials H1, H2 and H3. Porosity was assessed after every 10 cycles for 50 cycles.

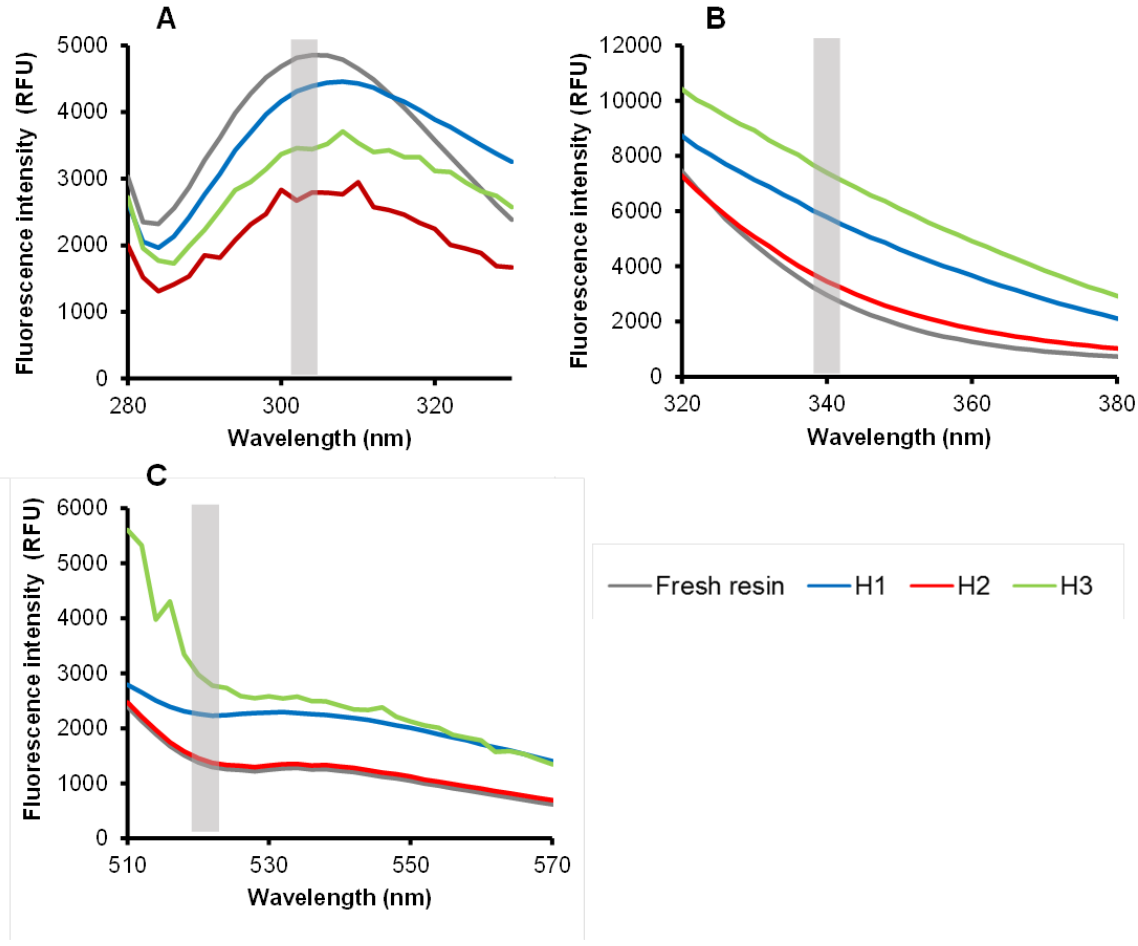


Figure 4: Fluorescence intensity analysis of cycled resins for the detection of residual material. Fluorescence analysis was performed on resin after 50 chromatographic cycles with feed H1, H2 or H3. Fresh resin was also analysed as the control. Excitation wavelengths were varied for each target moiety and the emission spectra recorded. (A) Protein A ligand density targeted (emission 303 nm), excitation 260 nm; (B) HCP and mAb deposition targeted (emission 340 nm), excitation 280 nm; (C) DNA deposition targeted (emission 520 nm), excitation 480 nm.

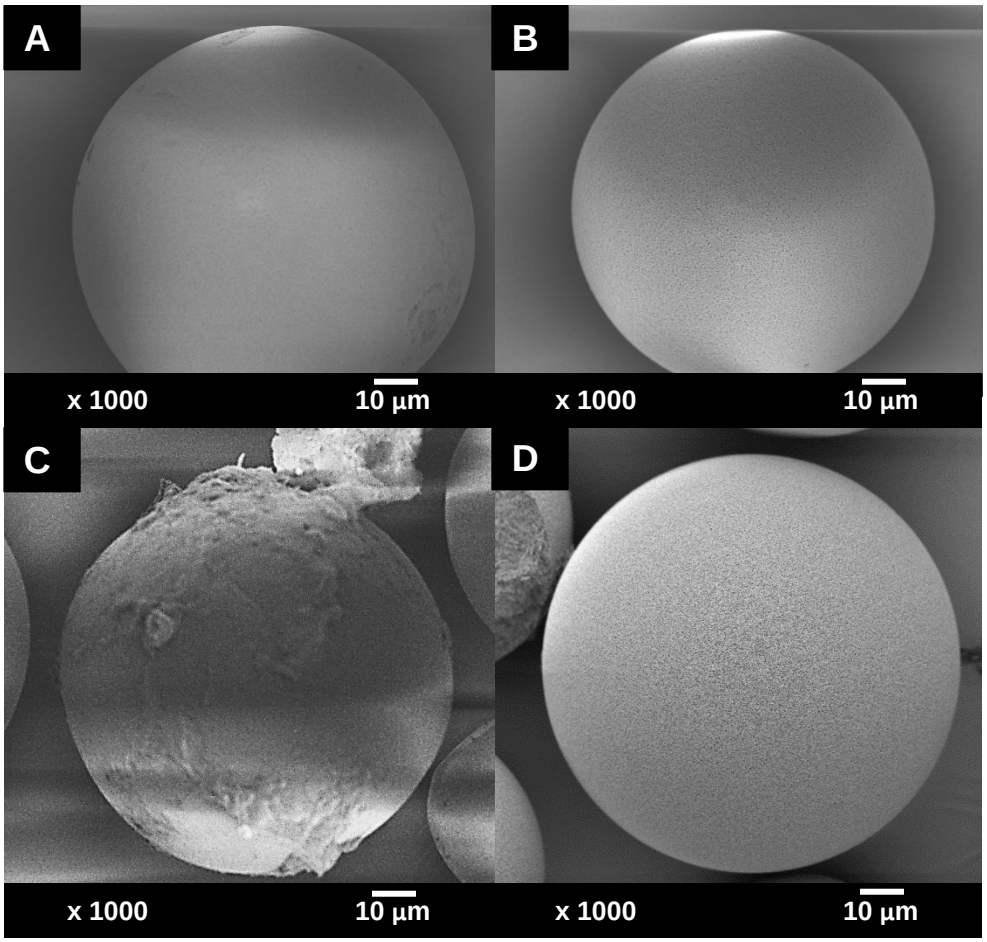


Figure 5: SEM images of resin cycled 50 times with different feed materials. (A) H1, (B) H2, (C) H3, (D) Fresh resin. Images are representative of at least three resin beads per feed material condition.

Table 1: Components of feed materials loaded onto columns. Values normalized to quantities loaded per chromatographic cycle. Percentage standard error inferred from analytical standards was as follows: mAb titer 0.05%; histones 1.7%; Non-histone HCP 2.29%; DNA 3.4%; protease 1.9%.

Feed material	mAb titer (mg/ml)	Histone (µg / ml resin)	Non-histone (mg / ml resin)	DNA (µg / ml resin)	Protease (µg / ml resin)
H1	4.96	0.7	1.4	0.6	0.095
H2	1.05	0.1	1	3.6	0.150
H3	0.98	1.5	1.2	2.2	0.110

Table 2: Histone and non-histone HCP content in the protein A eluate.

Feed material	Cycle number	Histone (ng/ml)	Other HCP (ng/ml)
H1	1	27.5	220
H1	50	84.5	220
H2	1	9.3	180
H2	50	6.4	160
H3	1	19.4	180
H3	50	39.0	150

Table 3: Residual on-resin histone concentration. Resin samples from each feed material condition were digested after 50 cycles and assayed for histones using ELISA. Concentrations normalized to micrograms per ml of resin used in the column.

Feed material	Histone (µg / ml resin)
H1	3.8
H2	0.4
H3	9.4

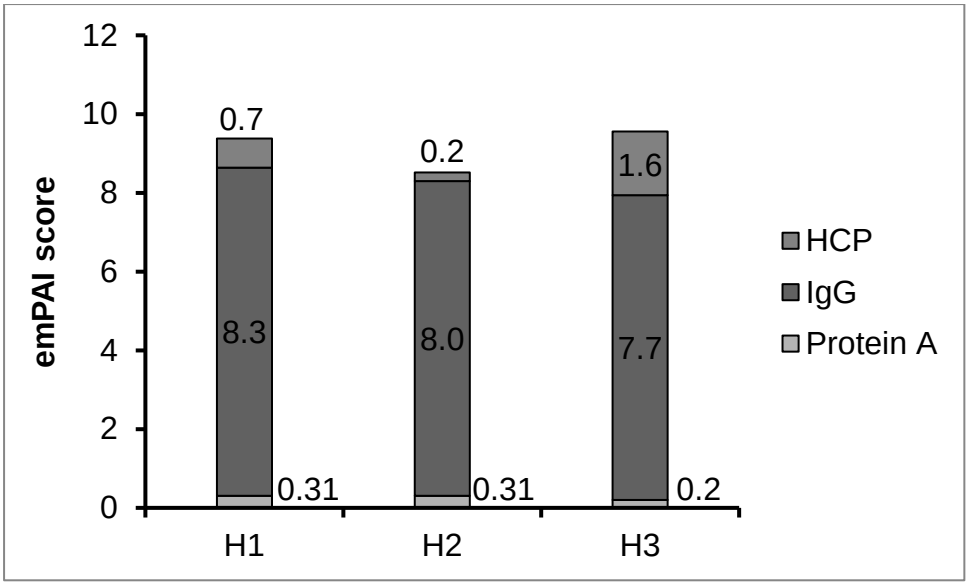


Figure 6 – Proteinaceous material recovered from resin cycled with different feed materials. Resins underwent 50 cycles and samples analyzed with LC-MS/MS. Statistically relevant peptide matches and the corresponding emPAI scores were grouped and plotted for each feed material. Process contaminants (trypsin, human keratin) were excluded from analysis.

References

[1] D.E. Steinmeyer, E.L. McCormick, Drug Discov Today 13 (2008) 613.

[2] P. Gagnon, E. Grund, Biopharm-the Technology & Business of Biopharmaceuticals 9 (1996) 54.

[3] A.A. Shukla, B. Hubbard, T. Tressel, S. Guhan, D. Low, J Chromatogr B Analyt Technol Biomed Life Sci 848 (2007) 28.

[4] A.S. Rathore, M. Pathak, G.J. Ma, D.G. Bracewell, Biopharm International 28 (2015) 28.

[5] B. Kelley, Biotechnol Prog 23 (2007) 995.

[6] J.E. Hale, D.E. Beidler, Anal Biochem 222 (1994) 29.

[7] R. Hahn, K. Shimahara, F. Steindl, A. Jungbauer, Journal of Chromatography A 1102 (2006) 224.

[8] Z. Yan, J. Huang, J Immunol Methods 237 (2000) 203.

[9] C.P. Jiang, J. Liu, M. Rubacha, A.A. Shukla, Journal of Chromatography A 1216 (2009) 5849.

[10] K. Lintern, M. Pathak, C.M. Smales, K. Howland, A. Rathore, D.G. Bracewell, Journal of Chromatography A 1461 (2016) 70.

[11] P. Gagnon, R. Nian, J. Lee, L. Tan, S.M. Latiff, C.L. Lim, C. Chuah, X. Bi, Y. Yang, W. Zhang, H.T. Gan, J Chromatogr A 1340 (2014) 68.

[12] P. Gagnon, R. Nian, L. Tan, J. Cheong, V. Yeo, Y. Yang, H.T. Gan, J Chromatogr A 1374 (2014) 145.

[13] P. Gagnon, R. Nian, Y.S. Yang, Q.H. Yang, C.L. Lim, Journal of Chromatography A 1408 (2015) 151.

[14] L. Tan, V. Yeo, Y. Yang, P. Gagnon, Anal Bioanal Chem 407 (2015) 4173.

[15] S. Zhang, W. Daniels, J. Salm, J. Glynn, J. Martin, C. Gallo, R. Godavarti, G. Carta, Biotechnol Bioeng 113 (2016) 141.

[16] M. Pathak, A.S. Rathore, J Chromatogr A 1459 (2016) 78.

[17] J.N. Carter-Franklin, C. Victa, P. McDonald, R. Fahrner, J Chromatogr A 1163 (2007) 105.

[18] R.L. Fahrner, D.H. Whitney, M. Vanderlaan, G.S. Blank, Biotechnol Appl Biochem 30 (Pt 2) (1999) 121.

18 - Method for monitoring of foulants present on chromatographic resins using fluorescence probe", application 201611015421, filed 3rd May 2016 at Indian Patent Office.

19 - Pathak M, Lintern K, Chopda V, Bracewell DG, Rathore AS. Fluorescence based real time monitoring of fouling in process chromatography. Scientific Reports. 2016. in print.